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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Johnson et al.

Application No.: 08/458,019

Group Art Unit: 1651

Filed: 1 June 1995

Examiner: Lilling

For: PROCESSES FOR IN VIVO PRODUCTION OF  
ASTAXANTHIN AND PHAFFIA RHODOZYMA  
YEAST OF ENHANCED ASTAXANTHIN CONTENT

DECLARATION UNDER 37 CFR §1.131

Assistant Commissioner of Patents  
Washington, D.C. 20231

Sir:

We, the undersigned co-inventors, do hereby declare and state:

1. We are co-inventors of claims 25-34 currently pending in U.S. Ser. No. 08/458,109 and of the subject matter described therein.

2. Prior to 15 April 1988, the invention embodied in claims 25-34 of U.S. Ser. No. 08/458,019 was reduced to practice in the United States.

3. Prior to 15 April 1988, practicing the methods provided

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U.S. Appln. No. 08/458,019

in the above-captioned application, numerous strains of Phaffia which overproduced astaxanthin at levels above that found in wild type Phaffia were obtained, thereby attesting to the reproducibility of the methods and teachings described in the above-captioned application.

4. Thus, wild type or astaxanthin-overproducing strains of Phaffia were treated as taught in the above-captioned application to obtain strains producing higher and higher levels of astaxanthin.

5. The initial screenings of the strains often were visual because the feature of interest is a pigment which is manifest as culture color.

6. Prior to 15 April 1988, numerous strains were prepared, propagated as provided in the above-captioned application and then the amount of astaxanthin produced by each strain was determined as provided in the above-captioned application.

7. Attached hereto are copies of pages from laboratory notebooks depicting representative experiments demonstrating the reduction to practice of the invention as claimed.

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a. Exhibit A is a copy of a page from a Kwok Ho laboratory notebook setting forth the results of astaxanthin content in Phaffia exposed to variety of treatments in shake flask cultures. The right-most column of figures --747, 865 and so on-- relate to astaxanthin content in micrograms per gram. All of the cultures contained astaxanthin at a concentration of more than 700 micrograms per gram.

b. Exhibit B is a copy of a page from a laboratory notebook of Beril Geldiay-Tuncer setting forth the results of astaxanthin content in Phaffia astaxanthin mutants. Ant 1-4 is a deposited strain. As provided in the record, Ant 1-4 is identified as IGI887J2 in the application; K20 is identified as IGI887J1 in the application; and Ant 1-460 is identified as IGI2880B60 in the application. Numerous strains produced astaxanthin at a concentration of more than 700 micrograms per gram.

c. Exhibit C is a copy of a page from a laboratory notebook of Beril Geldiay-Tuncer setting

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forth the results of astaxanthin content in Phaffia astaxanthin mutants. Ant 1-460 is identified as IGI2880B60 in the above-captioned application. Numerous strains produced astaxanthin at a concentration of more than 700 micrograms per gram.

d. Exhibit D is a copy of a page from a laboratory notebook of Beril Geldiay-Tuncer setting forth the results of astaxanthin content in Phaffia astaxanthin mutants. Ant 1-4 is a deposited strain. Ant 1-4 is identified as IGI887J2 in the application; K20 is identified as IGI887J1 in the application and Ant 1-460 is identified as IGI2880B60 in the application.

8. The dates redacted from the attached copies of laboratory notebook pages relating to testing various Phaffia strains for astaxanthin content are prior to 15 April 1988.

9. All of the activities described therein occurred in the United States.

10. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information

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and belief are believed to be true; and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the instant application or any patent issuing therefrom.

Further Declarants sayeth not.

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Date

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Eric A. Johnson

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Date

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Huei-hsiung Yang

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Date

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Beril Geldiay-Tuncer

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Date

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William T. Hall

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Date

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David Schreiber

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Date

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Kwok Ho

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Further Declarants sayeth not.

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Date

Eric A. Johnson

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Date

Huei-hsiung Yang

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Date

Beril Geldiay-Tuncer

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04.01.99

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Date

William T. Hall

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Date

David Schreiber

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Date

Kwok Ho

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Further Declarants sayeth not.

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Date

Eric A. Johnson

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Date

Huei-hsiung Yang

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Date

Beril Geldiay-Tuncer

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Date

William T. Hall

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Date

David Schreiber

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4/1/99

Date



Kwok Ho

(JL)

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Further Declarants sayeth not.

Date

April 1, 1999

Date

Eric A. Johnson

Huei-hsiung Yang

Beril Geldiay-Tuncer

Date

William T. Hall

Date

David Schreiber

Date

Kwak Ho

## Phaffia Strain Stock (S)

Stock No.

Purpose: Precursor &amp; screen #2

Method: Cell batch is from PHA125 (200L), age 49 hrs., 2.5mls were added to each flask,

Stock (Total vol) (Total l/w added to 25 ml flask)

	Dry wt.	T.I (ug/l)
1. squal 0.5ml	3.9.3	747
2 squal 0.2ml	32.7	863
3 DL methionine(1%) .05g	26.7	1138
4. DL methione (.08%) .02g	27.4	1113
5. Ac acetate (.08%) .02g	27.77	1201
6. Na acetate (.4%) .025g	2.8.9	1052
7. Na propionate (.08%) .02g	24.9	1115
8. Na propionate (.4%) .025g	23.1	891
9. safflower oil 0.3ml	34.6	913
10 peanut oil 0.3ml	33.3	846
11 lecithin (.2%) .05g	27.4	1000
12 lecithin (.08%) .02g	27.2	1143
13 Vitamin solution .05ml	27.3	1154
14 control	25.9	1158

Written

Chlorinated by me:

Date

Inventorized by

Documented by

Date

EXHIBIT A

To Page 11a

Purpose: Comparison of astaxanthin titer's within mutants K-20, Ant 1:4, BK 2, ~~28 cont~~, Ant 1-428, and Ant 1-460 in YM broth.

Results: I.D. - Part # Pan# Pan+cells 8/5ml A<sub>475nm</sub> patch (fl) <sup>ASTAXANTHIN</sup> (ug/1g)

1- K-20	0-560	10	952
2- K-20	0-546	10	978
1- BK 2	0-537	10	1081
2- BK 2	0-513	10	1035
1- Ant 1-418	0.499	10	770
2- Ant 1-428	0.441	10	870
1- Ant 1-460	0.737	11	1608
2- Ant 1-460	0.801	11	1678
1- Ant 1-4	0.417	10	661
2- ant 1-4	0.435	10	670

Ant 1-424	0.470	10	777
Ant 1-429	0.446	10	624
Ant 1-439	0.457	10	680
Ant 1-433	0.463	10	648

As seen above ant 1-460 is still pretty high. A stock culture of this new mutant will be prepared for the fermentation department.

To Page 11a

Witnessed	Understood by me,	Date	Invented by <u>Bentzema</u>	Date
			Recorded by <u>Bentzema</u>	

EXHIBIT B

Screening:

Book No.

31

Purpose: Screening of some of the selected mutant colonies of Ant 1-460 (NTG mutagenesis) in YM.

Incubation period 5 days.

Results: ml. per culture used for all the below was 20ml.

I.D.	Pan #	Pan (up)	Pan + cells	% Salicin	Aman (±)	Asterisk
Ant 1-460 (parent)	36	0.91607	1.02366	.027	0.338	1192
Ant 1-4601	30	0.99679	1.02822	.031	0.357	1096
Ant 1-4602	40	0.77640	1.02643	.03	0.344	1092
Ant 1-4604	33	0.97272	1.02488	.032	0.402	1196
Ant 1-4605	35	0.99151	1.02348	.031	0.380	1167
Ant 1-4606	38	1.00155	1.03178	.01	0.353	1096
Ant 1-4607	21	1.00556	1.03960	.032	0.366	1077
Ant 1-4608	37	1.79737	1.02785	.030	0.421	1336
Ant 1-4609	39	0.91705	1.02759	.03	0.408	1295
Ant 1-46000	31	1.00324	1.03379	.034	0.353	974
Ant 1-46011	34	0.99860	1.02753	.03	0.345	1085

Ant 1-4608 and Ant 1-4607 looks promising  
Check these back again.

Cleanness of the strains were was tested  
by plating some on to YM weighed before extraction  
of the 5 day old cultures.

Witness	I understand by me,	Date	Invented by	Bent Hansen	Date	To Page No.
			Recorded by	Bent Hansen		

EXHIBIT C

Pigment formation w/ respect to time Book No.

32

Page 16

Purpose: To observe how and when the pigment astaxanthin is produced and does it decrease with time after 5 days. This has been a question because of variances we have been getting with 3 day old cultures and 5 to 6 day old cultures, astaxanthin quantities.

Method: Brothum: Frozen Capped Stock of BK2, Ant-4, K-20, and 1-460

Medium YM in 500 ml Capped Flasks w/  
60ml volume  
10ml (5 for dry wt, 5 for astaxanthin)  
Take sample on 3rd, 5th and 7th day for  
quantification.

Results:

TIME → I.D.	48 hr.	72 hr.	144 hr.	
	concentration (mg/l)	↓	↓	↓
BK2	623.91	850	1090	
K-20	635.80	914.7	1017	
Ant-4	496.7	499	610	
And 1-460	658.10	809.5	1509	

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Instructor Understood by me:	Date	Invented by <u>Berthune</u>	Date
		Recorded by <u>Berthune</u>	

EXHIBIT D